Pol-Specific CD8⁺ T Cells Recognize Simian Immunodeficiency Virus-Infected Cells Prior to Nef-Mediated Major Histocompatibility Complex Class I Downregulation[▽]

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Effective, vaccine-induced CD8⁺ T-cell responses should recognize infected cells early enough to prevent production of progeny virions. We have recently shown that Gag-specific CD8⁺ T cells recognize simian immunodeficiency virus-infected cells at 2 h postinfection, whereas Env-specific CD8⁺ T cells do not recognize infected cells until much later in infection. However, it remains unknown when other proteins present in the viral particle are presented to CD8⁺ T cells after infection. To address this issue, we explored CD8⁺ T-cell recognition of epitopes derived from two other relatively large virion proteins, Pol and Nef. Surprisingly, infected cells efficiently presented CD8⁺ T-cell epitopes from virion-derived Pol proteins within 2 h of infection. In contrast, Nef-specific CD8⁺ T cells did not recognize infected cells until 12 h postinfection. Additionally, we show that SIVmac239 Nef downregulated surface major histocompatibility complex class I (MHC-I) molecules beginning at 12 h postinfection, concomitant with presentation of Nef-derived CD8⁺ T-cell epitopes. Finally, Pol-specific CD8⁺ T cells eliminated infected cells as early as 6 h postinfection, well before MHC-I downregulation, suggesting a previously underappreciated antiviral role for Pol-specific CD8⁺ T cells.

More than 25 million people have died from human immunodeficiency virus (HIV)/AIDS since 1981, making the epidemic one of the deadliest in history. As such, the development of a prophylactic vaccine remains one of the world's top health priorities. Unfortunately, conventional vaccines against HIV have failed, highlighting the need for novel vaccine approaches. Multiple studies have demonstrated the important role of CD8⁺ T cells in controlling HIV and simian immunodeficiency virus (SIV) infections (12, 21, 25, 26), and, accordingly, research is now under way to develop vaccine modalities which elicit effective CD8⁺ T cells. However, there is no clear consensus as to which viral proteins should be used for vaccination. For maximum antiviral activity, vaccine-induced CD8⁺ T cells should recognize infected cells early enough to prevent the production of progeny virus. We previously reported that Gag-specific but not Env-specific CD8⁺ T cells were able to recognize epitopes from virion-derived proteins as early as 2 h after infection (33). Whether any other proteins present in the viral particle, such as Pol and Nef, can be presented early after infection is not known.

The *pol* gene, which lacks an initiation codon, lies in the -1 reading frame with respect to *gag*. As a result, Pol is synthesized only as part of a Gag-Pol fusion protein at a ratio of 1:20

to Gag (16). The 160-kDa Gag-Pol protein contains the *pol*-encoded transframe (TF) protein and the essential viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN), which are efficiently packaged into the viral particle. Through an interaction with the C terminus of Nef, the TF protein of the Gag-Pol precursor in turn packages Nef into the virion (8).

In addition to the three essential genes common to all retroviruses, the primate lentiviruses HIV and SIV harbor the auxiliary gene *nef*. Unlike the *gag*, *pol*, and *env* genes, *nef* is dispensable for viral replication in vitro. Although *nef* may not play a direct role in viral replication, its persistence implies a role in viral pathogenesis. Indeed, infection with *nef*-defective viruses correlates with attenuated disease in HIV-infected humans (20, 31) and SIV-infected macaques (9, 18).

Translation of the *nef* gene gives rise to a myristoylated 27-kDa protein with a diverse array of functions. Nef enhances viral particle infectivity (27), activates infected cells to promote viral replication (3), and downregulates multiple transmembrane proteins including CD3 (4), CD4 (2), and major histocompatibility complex class I (MHC-I) (35). Despite the multiple activities of Nef, MHC-I downregulation is one of the most important Nef-mediated immune evasion mechanisms. Virus-specific CD8⁺ T cells, which play a critical role in containing viral replication in both the acute (21) and chronic (12, 26) phases of disease, exert their antiviral effect by recognizing MHC-I-bound viral epitopes. By decreasing the number of MHC-I molecules available to present viral epitopes, Nef interferes with the ability of CD8⁺ T cells to suppress viral replication (40, 49). Perhaps most importantly, Nef-mediated

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MHC-I downregulation shields infected cells from CD8⁺ T-cell-mediated killing in vitro (7). Furthermore, mutations in SIV *nef* which selectively ablate its ability to downregulate MHC-I revert in vivo after inoculation into macaques (28, 38), highlighting the importance of this function.

The kinetics of Nef-mediated MHC-I downregulation in relation to the presentation of CD8⁺ T-cell epitopes remain undefined. Nef diminishes the effectiveness of CD8⁺ T cells after it is expressed (7). Therefore, it is possible that CD8⁺ T-cell responses capable of recognizing infected cells prior to Nef-mediated MHC-I downregulation may be more effective than responses directed against viral antigens that are expressed later in infection. However, it is not known when Nef downregulates MHC-I expression.

To address these issues, we assessed how soon after infection CD8⁺ T cells recognized epitopes derived from Pol and Nef. Here we show that Pol-derived epitopes appeared on the cell surface within 2 h of infection. In contrast, Nef-derived epitopes did not appear until 12 h postinfection. Additionally, we monitored surface MHC-I expression in synchronously infected cells throughout the viral replication cycle. Nef-mediated MHC-I down-modulation commenced at 12 h postinfection, simultaneous with the presentation of Nef epitopes.

MATERIALS AND METHODS

Animals. The animals used in this study were Indian rhesus macaques (*Macaca mulatta*) from the Wisconsin National Primate Research Center colony. They were typed for the MHC-I alleles *Mamu-A*01* and *Mamu-B*17* by sequence-specific PCR as described elsewhere (17). Animals were also typed for *Mamu-A*07* as described elsewhere (M. Kaizu et al., unpublished data). The animals were cared for according to the regulations and guidelines of the University of Wisconsin Institutional Animal Care and Use Committee.

Cell isolation and culture. Target cells were generated from freshly isolated peripheral blood mononuclear cells (PBMC) obtained from SIV-naïve Indian rhesus macaques using Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) density centrifugation. CD4⁺ T cells were isolated using CD4 microbeads and LS columns purchased from Miltenyi Biotec and used according to the manufacturer's instructions. Targets were activated by incubation overnight with staphylococcal enterotoxin B (2.5 µg/ml) and antibodies to CD28 (2.5 µg/ml), CD3 (2.5 µg/ml), and CD49d (2.5 µg/ml) and cultivated in the presence of 100 U interleukin-2 (IL-2)/ml. CD4⁺ targets were consistently ≥95% CD3 and CD4 positive. For MHC-I kinetic experiments, CD4⁺ targets were activated with concanavalin A (10 µg/ml) up to 72 h prior to infection. Virus-specific CD8⁺ T-cell clones were generated as previously described (6). Briefly, three rounds of limiting dilution were performed on CD8-enriched PBMC from SIV-infected macaques with repeated stimulations of autologous B-lymphoblastoid cell lines pulsed with the peptide of interest. Purity of clones was verified by tetramer and/or intracellular cytokine staining (ICS) assays. Cells were cultured in R15-100 (RPMI 1640 containing 15% fetal calf serum and 100 U/ml IL-2). IL-2 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Generation of SIVmac239 and synchronized infections. SIVmac239 was generated (11) and purified (32) as previously described. Briefly, Vero cells were transfected with plasmid DNA encoding proviral sequences. CEMx174 cells were added to the Vero cultures, and the virus was subsequently expanded on CEMx174 cells. Cell-free supernatant was collected 2 days after peak syncytium formation. The virus was subsequently purified through a 20% sucrose cushion immediately prior to use in the kinetic ICS (KICS) assay. ViroMag beads were obtained from OZ Biosciences (Marseille, France). SIVmac239 was magnetized with ViroMag beads, and cells were synchronously infected according to the manufacturer's instructions at a multiplicity of infection (MOI) of 1. Briefly, target cells were incubated with the magnetized virus for 15 min in the presence of a magnetic field, washed once in phosphate-buffered saline (PBS), washed once with 0.25 µg/ml trypsin-EDTA (Sigma Aldrich) to remove surface-bound virions (34), and then washed two more times with PBS. Identical results were obtained in all assays when the trypsin-EDTA wash was excluded (data not shown). Infected targets were then incubated in R15-100.

To measure 50% tissue culture infectious dose (TCID₅₀), activated, primary CD4⁺ T cells were infected with 10-fold dilutions of sucrose-purified virus using the magneticococfection technique as described above. Titer was determined from the lowest virus concentration that gave rise to infected cultures 8 days after infection, as determined by Gag p27 enzyme-linked immunosorbent assay (Zepto-Matrix Corporation). The magnetic-infection technique resulted in an approximate 10-fold increase in TCID₅₀ levels compared to a standard 4-h infection (data not shown).

2,2'-Diithiodipyridine (aldrithiol-2 [AT-2])-inactivated SIVmac239 was a gift from Jeffrey D. Lifson and was produced from infected SUPT1-CCR Cl 30 cells essentially as described previously (23). Cells treated with tenofovir were incubated with 400 µM tenofovir for at least 2 h prior to infection and throughout the experiment. Tenofovir disoproxil fumarate was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

VSA. We performed in vitro viral-suppression assays (VSAs) as previously described (24). Briefly, CD8-depleted, phytohemagglutinin-stimulated lymphocytes were infected with SIVmac239 at an MOI of 5×10^{-5} and incubated with SIV-specific CD8⁺ T-cell clones for 7 days. At the end of the assay (day 7), viral RNA copy measurement and intracellular Gag p27 staining were performed on the coculture to measure SIVmac239 infection.

Twenty-four-hour elimination assay. Synchronously infected CD4⁺ T lymphocytes (1×10^5) were infected as described above and cocultured with CD8⁺ T-cell clones at an effector/target (E/T) ratio of 1:1 in ~150 µl of R15-100 at 37°C. At specific time points, the wells were harvested and stained with antibodies specific for CD4 and CD8 (BD Biosciences), followed by intracellular Gag p27 staining using Fix and Perm (CALTAG, Burlingame, CA) according to the manufacturer's protocol with fluorescently conjugated 55-2F12 Gag p27 antibody (NIH AIDS Research and Reference Reagent Program, Germantown, MD) at ~0.75 mg/ml. CD8⁺ T-cell clones used in this assay responded similarly to B-lymphoblastoid cell lines pulsed with cognate antigen and suppressed viral replication in a longer 7-day VSA (6).

ICS assay. The ICS assay was performed as described previously (46) with some modification. Briefly, 1×10^5 synchronously infected CD4⁺ targets were incubated with 1×10^5 SIV-specific CD8⁺ clones for 1.5 h at 37°C in 200 µl of R15-100 with anti-CD28 and anti-CD49d antibodies (BD Biosciences). To prevent protein transport from the Golgi apparatus, 10 µg of brefeldin A per ml was added at specific time points postinfection and the cells were incubated a further 5 h at 37°C. Cells were washed and stained for surface expression of CD4 and CD8 markers and fixed overnight in 1% paraformaldehyde at 4°C. The following day, cells were permeabilized in buffer containing 0.1% saponin and stained for gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) before being fixed in 1% paraformaldehyde for 2 h at 4°C. Events were collected on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with CellQuest software and analyzed with FlowJo version 8.1.0 or above for Macintosh (Tree-star, Ashland, OR). A positive result is defined as being at least twofold higher than the negative control.

RESULTS

Pol-derived epitopes are presented early after infection. The longer an epitope is presented by an MHC-I molecule on the surface of an infected cell, the more likely it is to serve as an effective target for antiviral CD8⁺ T cells. Accordingly, it has been hypothesized that CD8⁺ T cells directed against epitopes derived from the early proteins Tat, Rev, and Nef might be more effective than CD8⁺ T cells which recognize epitopes from the late structural proteins Gag, Pol, and Env (25, 42, 43, 48). However, we recently demonstrated that the incoming virion can itself be a source of antigen, as virion-derived Gag epitopes are present on the surfaces of CD4⁺ T lymphocytes by 2 h postinfection (33).

To assess when Pol- and Nef-derived epitopes are presented to CD8⁺ T cells by MHC-I molecules on the surfaces of infected CD4⁺ T lymphocytes, we performed the KICS assay (33) using CD8⁺ T-cell clones specific for Pol and Nef (Table 1). Surprisingly, in three independent assays, we observed robust MHC-I-restricted recognition of the Pol PR₁₄₇₋₁₅₆-derived epitope, LV10, at 6 h postinfection, when no Nef-derived

TABLE 1. SIV-specific CD8⁺ T-cell clones used in the study

Clone	Protein	Amino acid position	Epitope	MHC restriction	Sequence	No. of clones used
Pol LV10	Pol p10 PR	147–156	LV10	A*01	LGPHYTPKIV	5
Pol YL8	Pol p31 IN	782–789	YL8	A*07	YHSNVKEL	2
Nef IW9	Nef	165–173	IW9	B*17	IRYPKTFGW	3

epitopes were detectable on the surfaces of infected cells (Fig. 1A). Interestingly, recognition of the Pol-derived epitope was biphasic, with a second wave of Pol epitope presentation occurring between 18 and 24 h postinfection.

To confirm our finding that Pol-derived epitopes are presented early after infection, we defined a new, previously unreported Pol CD8⁺ T-cell epitope, Pol IN_{782–789} YL8, restricted by *Mamu-A*07* (Table 1) (J. Reed et al., unpublished data). Using CD8⁺ T-cell clones specific for this new Pol

epitope in the KICS assay, we again observed early recognition of infected cells (Fig. 1B). In contrast, Nef-specific CD8⁺ T-cell clones first recognized infected cells at 12 h postinfection, with recognition increasing over the course of the assay (Fig. 1C). Importantly, infected MHC-I-mismatched targets did not activate the CD8⁺ T-cell clones at any time point during the assay, indicating that cytokine secretion was MHC-I restricted and epitope specific.

To determine exactly when Pol-derived epitopes are present on the surfaces of infected cells, we next examined the first 6 h after infection in greater detail. Both Pol-specific CD8⁺ T-cell clones recognized infected cells by 2 h postinfection (Fig. 2A and B). In contrast, Nef-specific CD8⁺ T-cell clones failed to recognize infected CD4⁺ T lymphocytes during the first 7 h of infection although they recognized the same infected cells at 24 h postinfection (Fig. 2C and data not shown). Two different Pol-specific CD8⁺ T-cell clones, restricted by two separate MHC-I molecules, recognized infected cells by 2 hours postinfection. These data indicate that early presentation of Pol-derived epitopes occurs with at least two Pol proteins, Pol PR and Pol IN.

Pol but not Nef epitopes can be virion derived. We previously demonstrated that Gag-specific CD8⁺ T cells recognize infected cells early after infection because the infected cells present epitopes derived from the incoming, virion-associated Gag protein (33). Therefore, we hypothesized that the early presentation of Pol epitopes was due to Pol proteins in the infecting virus particle being presented by the infected cells. To test this hypothesis, we repeated the KICS assay using noninfectious, AT-2-inactivated SIV or infectious virus in the presence of the reverse transcriptase inhibitor tenofovir. Under these conditions, where de novo synthesis of viral proteins is blocked, Pol-specific CD8⁺ T cells still efficiently recognized infected cells at 6 h postinfection in three independent assays (Fig. 3A). However, the second wave of the biphasic Pol presentation seen in earlier experiments with infectious virus (Fig. 1A and B) was ablated. This indicates that the early wave of antigen presentation was likely due to virion-derived Pol proteins, while the second wave of epitope presentation, which began between 18 and 24 h postinfection, was dependent upon de novo protein synthesis of the Pol protein.

Although early Pol-specific recognition occurred in the absence of de novo protein synthesis, recognition of infected cells by Nef-specific CD8⁺ T cells was completely ablated (Fig. 3B). We observed no downregulation of surface CD4 or MHC-I at any time after infection in cells pulsed with AT-2-inactivated SIV or infected with SIVmac239 in the presence of tenofovir (data not shown). Therefore, no detectable de novo synthesis of Nef was occurring. This indicated that Nef-specific CD8⁺ T cells recognized infected cells only after de novo synthesis of Nef. Thus, although Nef is efficiently packaged into the virus

FIG. 1. Pol-specific CD8⁺ T cells recognize infected cells early after infection. MHC-I-matched and MHC-I-mismatched CD4⁺ T cells were synchronously infected and cocultured at an E/T ratio of 1:1 with CD8⁺ T-cell clones specific for (A) Pol LV10, (B) Pol YL8, or (C) Nef IW9. Results are shown as the percentages of maximum cytokine staining of TNF- α and IFN- γ detected during the assay. Typical percentages of maximum cytokine staining were ~20 to 40%. Data shown are means $\pm$ standard deviations for duplicate samples and indicative of at least three independent experiments.

FIG. 2. Pol-specific CD8⁺ T cells recognize infected cells by 2 hours postinfection. MHC-I-matched and MHC-I-mismatched CD4⁺ T cells were synchronously infected and cocultured at an E/T ratio of 1:1 with CD8⁺ T-cell clones specific for (A) Pol LV10, (B) Pol YL8, or (C) Nef IW9. Results are shown as the percentages of maximum cytokine staining of TNF- α and IFN- γ detected during the assay. Data shown are means $\pm$ standard deviations for duplicate samples and indicative of at least three independent experiments.

particle (29, 45), it does not appear that CD8⁺ T-cell epitopes are derived from Nef proteins present in the incoming virion.

Nef downregulates MHC-I at 12 h postinfection. Although it is well established that Nef antagonizes CD8⁺ T-cell activity by decreasing the density of MHC-I molecules on the surfaces of infected cells (7), no data exist on the kinetics of this event. To determine when Nef begins to down modulate surface expression of MHC-I, we synchronously infected primary CD4⁺ T lymphocytes with SIVmac239 and measured MHC-I levels on

the surfaces of infected and uninfected cells throughout the replication cycle.

Immediately after a synchronized infection, Gag p27 is detected in infected cells, which appear in the CD4⁺ and Gag p27⁺ gate (Fig. 4A). Following these cells throughout the course of the infection, we first detected Nef-mediated downregulation of CD4 at 12 h postinfection (Fig. 4A). However, a small subset of the infected cells never experienced CD4 or MHC-I downregulation. We hypothesize that cells in the

FIG. 3. Pol, but not Nef, epitopes can be virion derived. CD4⁺ T lymphocytes were infected with 350 ng Gag p27 of either AT-2-inactivated SIVmac239 or infectious SIVmac239 in the presence of 400 μ M tenofovir and cocultured with CD8⁺ T cells specific for (A) Pol LV10 or (B) Nef IW9. Cells infected with infectious SIVmac239 were treated with 400 μ M tenofovir for at least 2 hours prior to infection and throughout the experiment to inhibit reverse transcription. Data shown are means $\pm$ standard deviations for duplicate samples and indicative of at least three independent experiments.

FIG. 4. Nef downregulates MHC-I at 12 h postinfection. (A) Gating strategy used to demarcate productively infected (Gag p27⁺ CD4⁻) and uninfected (Gag p27⁻ and Gag p27⁺ CD4⁺) CD4⁺ T cells. (B) Kinetics of Nef-mediated MHC-I downregulation throughout the viral replication cycle. The gating strategies described above were used to monitor surface MHC-I levels in uninfected (black trace) and infected (gray trace) CD4⁺ T cells at the indicated time points postinfection. For time points prior to CD4 downregulation, the entire Gag p27⁺ population is used for the infected-cell trace. (C) MFI for MHC-I on uninfected and infected CD4⁺ T lymphocytes throughout the viral replication cycle. Results shown are means $\pm$ standard deviations for duplicate samples. Results are indicative of three independent experiments. (D) Intracellular Gag p27 and MHC-I surface stain of CD4⁺ T cells synchronously infected using SIVmac239 Y223F. At 24 h postinfection, MHC-I levels were monitored using the gating strategy described above.

CD4⁺ and Gag p27⁺ gate represented newly infected cells or cells that were infected with defective viruses, while cells in the CD4⁻ and Gag p27⁺ gate were productively infected. Therefore, to detect MHC-I downregulation, we compared surface levels of MHC-I on uninfected cells (CD4⁺ Gag p27⁻) versus productively infected cells (CD4⁻ Gag p27⁺). Using this gating strategy in three independent assays, we detected MHC-I downregulation first occurring at 12 h postinfection and then increasing throughout the course of the assay (Fig. 4B). When mean fluorescence intensity (MFI) of MHC-I staining on infected cells was compared to that on uninfected cells, we observed a similar pattern of downregulation (Fig. 4C).

Finally, to confirm that downregulation of MHC-I was Nef mediated, we repeated the assay using SIVmac239 Y223F, which contains a point mutation in a tyrosine residue required to downregulate MHC-I (37). At 24 h postinfection, we still

detected downregulation of CD4 but MHC-I downregulation was almost completely abolished (Fig. 4D). There was a slight decrease in surface MHC-I levels on infected versus uninfected cells (MFIs of MHC-I: 1,006 and 1,312, respectively), indicating that other viral proteins can, to a minor extent, downregulate MHC-I. However, the vast majority of MHC-I downregulation was Nef mediated.

Pol-specific CD8⁺ T cells eliminate infected CD4⁺ T lymphocytes early after infection in a 24-hour elimination assay. Although the KICS assay demonstrated that CD8⁺ T cells directed against Pol recognized infected cells early after infection, it remained unclear if Pol-specific CD8⁺ T cells could eliminate recently infected CD4⁺ T lymphocytes. Therefore, we next investigated whether CD8⁺ T cells directed against Pol could reduce the percentage of infected cells early after a synchronized infection. Since Gag p27 is detectable throughout

FIG. 5. Pol-specific CD8⁺ T cells eliminate infected cells early after infection. A Mamu-A*01⁺ Mamu-B*17⁺ CD4⁺ T-cell target was synchronously infected with SIVmac239 and cocultured at an E/T ratio of 1:1 with either (A) no CD8⁺ T cells or with CD8⁺ T cells specific for (B) Pol LV10 or (C) Nef IW9. (D) To ensure that elimination of infected targets was MHC-I dependent, a Mamu-A*01⁻ Mamu-B*17⁻ CD4⁺ T-cell target was synchronously infected and cocultured for 24 h with the CD8⁺ T-cell clones described above. Dot plots were generated by gating on live, CD8⁺ lymphocytes. Data are representative of four independent experiments and of three different Pol clones generated from two unrelated animals in different stages of disease progression (one elite controller and one progressor).

FIG. 6. Pol-specific CD8⁺ T cells suppress virus in the VSA. (A) Mamu-A*01⁺ Mamu-B*17⁺ or (B) Mamu-A*01⁻ Mamu-B*17⁻ CD8-depleted PBMC were infected at an MOI of 5×10^{-5} and incubated with either no CD8⁺ T cells or the indicated CD8⁺ T cells at an E/T ratio of 1:1 for 7 days. Percentages are percentages of Gag p27⁺ cells. (C) Concentrations of viral RNA (vRNA) in supernatant of VSA after 7 days of culture with MHC-I-matched targets. (D) Concentrations of vRNA in supernatant of VSA after 7 days of culture with MHC-I-mismatched targets. Data are representative of duplicate wells and of two independent experiments.

the entire viral replication cycle in infected cells (Fig. 4A), we measured elimination of infected cells as a reduction in the percentage of Gag p27⁺ cells.

Immediately after the synchronized infection, we detected Gag p27 in infected CD4⁺ T lymphocytes (Fig. 4A and 5A). After production of Nef, which downregulates surface CD4 expression at 12 h postinfection, Gag p27 is present in two distinct populations of infected cells, CD4⁺ and CD4⁻ T lymphocytes (Fig. 4A and 5). The Pol PR₁₄₇₋₁₅₆-specific clone, LV10, eliminated infected cells early after infection, reducing the frequency of Gag p27⁺ staining at 6 h postinfection from 41% (no-CD8⁺-T-cell control) to 31.7% (Fig. 5B). By 12 h postinfection, in four independent assays, there was a striking decrease in the percentage of Gag p27⁺ CD4⁻ cells in the cultures incubated with the Pol-specific clone, which continued throughout the course of the assay (Fig. 5A and B). These data suggest that CD8⁺ T-cell clones directed against Pol eliminated infected cells prior to Nef-mediated CD4 and MHC-I downregulation. In contrast, Nef-specific CD8⁺ T cells did not eliminate infected cells until after de novo synthesis of Nef (Fig. 5C). This agrees with our previous observation that virion-derived Nef proteins were not presented by MHC-I on the surfaces of infected cells. Finally, to ensure that elimination of infected CD4⁺ T lymphocytes was MHC-I restricted, we repeated the elimination assay using synchronously infected, MHC-I-mismatched targets. We observed no reduction of Gag p27 staining in MHC-I-mismatched targets, demonstrating that elimination of infected cells was MHC-I dependent (Fig. 5D).

Pol-specific CD8⁺ T cells suppress virus replication in the VSA. To further test our hypothesis that Pol-specific CD8⁺ T cells might be more effective than previously thought, we performed an in vitro VSA (25). In contrast to the 24-hour elimination assay, the VSA does not use a synchronized infection.

Instead, a smoldering infection is initiated with an extremely low MOI and allowed to spread for 7 days.

Both Nef IW9- and Pol LV10-specific CD8⁺ T-cell clones were effective in the VSA, reducing the frequency of infected cells by greater than 80% (Fig. 6A). Importantly, suppression was MHC-I mediated, as we observed no reduction of infected cells in MHC-I-mismatched targets (Fig. 6B). We previously demonstrated that Nef IW9-specific CD8⁺ T-cell clones are among the most potent CD8⁺ T cells tested in the VSA (6). In agreement with this, the Nef-specific CD8⁺ T-cell clone reduced the concentration of virus in the supernatant by nearly two logs (Fig. 6C). Although not as robust as the Nef-specific CD8⁺ T-cell clone, the Pol-specific CD8⁺ T-cell clone was still effective in the VSA and diminished the viral RNA copy number in the supernatant by over 1 log (Fig. 6C). Again, this suppression was MHC-I dependent, as no reduction in the viral RNA copy number was observed (Fig. 6D).

DISCUSSION

Here we show for the first time that presentation of Pol-derived CD8⁺ T-cell epitopes occurs by 2 h postinfection, well before MHC-I downregulation. In contrast, Nef-derived CD8⁺ T-cell epitopes did not appear on the surfaces of infected cells until 12 h postinfection. Since cells infected with chemically inactivated or inhibited viruses were recognized by Pol-specific but not Nef-specific effectors, early Pol epitopes were likely derived from the incoming virion. Nef epitopes, on the other hand, were derived only from de novo synthesis of the Nef protein, which resulted in MHC-I downregulation.

While it is possible that our synchronized in vitro assays use more virus than would be present during infections in vivo, two lines of evidence argue against this. First, a dose-response analysis determined that infection with 20 to 100 virions/cell

efficiently triggers presentation of incoming viral proteins (33). However, the vast majority of these infecting viral particles have been reported to be noninfectious (22, 30). Emerging evidence suggests that this may actually be an overestimation of defective virions and that ~ 1 in 10 viral particles is infectious (39). Even if we assume this higher estimate (1:10), then according to our dose-response analysis, infection with 2 to 10 infectious virions/cell is sufficient for presentation of incoming virion proteins. This level of infection is likely reached in vivo, especially in compartmentalized immune tissues such as lymph nodes, where there are concentrated bursts of localized infection (14). Secondly, Pol-specific CD8⁺ T cells were effective in the VSA, where the MOI was $\sim 10^5$ times smaller than that used in the synchronized in vitro assays. Therefore, Pol-specific CD8⁺ T cells were efficient in reducing infection in in vitro assays independent of whether a high or low MOI was used. We hypothesize that Pol-specific CD8⁺ T cells are effective due to the kinetic advantage gained by presentation of virion-derived epitopes.

Previous studies have demonstrated that low levels of Nef are sufficient to reduce CD4 surface expression while higher concentrations of Nef are required to remove MHC-I from the plasma membrane (36, 44). In contrast to these studies, we found that Nef-mediated downregulation of CD4 and downregulation of MHC-I occur simultaneously, indicating that similar levels of Nef are sufficient to down modulate these different targets. The discrepancy in these results likely stems from the different experimental systems used. The previous studies used transfection of a Nef-containing plasmid to drive Nef production, while we used infectious virus. Additionally, a recent report demonstrated that Nef-mediated MHC-I downregulation occurs early enough to interfere with the presentation of epitopes derived from early proteins, including CD8⁺ T-cell epitopes from Nef itself (1). In agreement with these data, we show that Nef diminished surface MHC-I beginning at 12 h postinfection, simultaneously with the presentation of Tat (33)- and Nef-derived epitopes.

Although both Pol and Nef are packaged into the virus particle (45), our CD8⁺ T-cell clones detected only Pol epitopes on the surfaces of infected cells by 2 h postinfection. Furthermore, CD4⁺ T cells infected with AT-2-inactivated SIV or treated with tenofovir still efficiently triggered Pol-specific but not Nef-specific CD8⁺ T cells. These results suggest that not all proteins in the virion are present in sufficient numbers to reach the threshold required to activate a CD8⁺ T cell. Indeed, Nef is present in significantly smaller quantities in the virus particle. An immature virus particle contains $>1,500$ molecules of Gag (47, 50), with an upper-limit estimate of $\sim 5,000$ Gag molecules (5). Because of the strict 20:1 ratio of Gag to Gag-Pol synthesis (16), there are 75 to 250 molecules of the Pol protein per virion. Nef is present in the virus particle at on the order of 10% of Pol RT, which corresponds to only 8 to 25 molecules of Nef per virion (45). This relatively small amount of virion-associated Nef is likely insufficient to produce enough peptide ligand for presentation by MHC-I molecules. Additionally, Nef is proteolytically cleaved by the viral protease in the maturing virus particle (10, 13), which might interfere with antigen processing. However, it has been demonstrated that HLA alleles that bind N-terminal dibasic peptides, such as HLA-B27, can present peptides from antigen present

in amounts insufficient for presentation by other MHC-I molecules (15). It would, therefore, be of interest to investigate whether HLA molecules capable of presenting lower quantities of antigen could present the small amount of Nef in the incoming virus particle.

These results are interesting in light of a recent study investigating the effect of Nef-mediated MHC-I downregulation on the ability of HIV-specific CD8⁺ T cells to suppress viral replication (41). Intriguingly, Pol-specific CD8⁺ T cells effectively eliminated HIV-infected cells, even in the presence of effective MHC-I down modulation. Our data agree with this study and offer a possible mechanism to explain why Pol-specific CD8⁺ T cells can effectively suppress viral replication in vitro in the face of Nef-mediated antagonism. By recognizing infected cells prior to Nef-mediated MHC-I interference, Pol-specific CD8⁺ T cells have a larger window of opportunity to eliminate infected cells than previously appreciated.

Our data further suggest that Pol may be a good target for vaccines designed to induce CD8⁺ T cells. However, as highlighted by a recent study of unvaccinated, chronically HIV-infected individuals (19), no correlations between Pol-specific responses and lower viral load have been observed. Reasons for this lack of correlation remain unclear. One explanation could be the level of antigen present during infection. There is 20-fold less Pol than Gag protein in a virus particle (5). Although virion-derived Pol epitopes are presented early after infection, the lower level of Pol antigen may be insufficient to induce effective CD8⁺ T cells directed against this protein in HIV-infected individuals. It is possible, therefore, that vaccine-induced Pol-specific CD8⁺ T cells might be more effective than those primed during acute HIV infection. It will be of interest to determine if vaccine-induced Pol-specific CD8⁺ T cells can significantly contribute to control of viral replication.

The longer an epitope is presented by an MHC-I molecule on the surface of an infected cell, the more likely it is to serve as an efficient target for CD8⁺ T cells. Pol-derived epitopes were presented by 2 h postinfection, and Pol-specific CD8⁺ T cells eliminated infected cells by 6 h postinfection, well before Nef-mediated MHC-I downregulation. This suggests that Pol-specific CD8⁺ T cells might contribute more to the antiviral CD8⁺ T-cell response than previously appreciated.

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